

# Solid-Phase Extraction and Reversed-Phase Ion-Pair High-Performance Liquid Chromatographic Determination of Chlorophacinone and Diphacinone Residues in Range Grass

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## Abstract

A reversed-phase ion-pair liquid chromatographic method is developed for the analysis of chlorophacinone and diphacinone in range grass. Chlorophacinone and diphacinone are extracted from range grass with chloroform, and the extracts are subjected to an aminopropyl solid-phase extraction procedure, concentrated, and analyzed by reversed-phase ion-pair high-performance liquid chromatography. Chlorophacinone and diphacinone are detected by ultraviolet absorption at 285 nm. The average recoveries from range grass fortified at 0.10, 1.0, and 10.0  $\mu\text{g/g}$  chlorophacinone and diphacinone are  $94\% \pm 3$  and  $97\% \pm 6$ . The limit of detection for chlorophacinone and diphacinone in range grass is estimated to be 0.015  $\mu\text{g/g}$  and 0.013  $\mu\text{g/g}$ , respectively.

## Introduction

Diphacinone (2-[diphenylacetyl]-1H-indene-1,3[2H]-dione) and chlorophacinone (2-[(chlorophenyl)phenylacetyl]-1H-indene-1,3[2H]-dione) belong to a group of compounds called indandiones. They are registered anticoagulant rodenticides commonly used to control populations of rats and mice. Chlorophacinone and diphacinone have acute oral lethal dosages with 50% mortality ( $\text{LD}_{50}$ ) dosages of 2 mg/kg for rats, compared with acute oral  $\text{LD}_{50}$  dosages of 50 mg/kg for anticoagulants such as warfarin and pindone. These anticoagulants are also effective in the control of other rodents such as pocket gophers (*Thomomys bottae*), Belding ground squirrels (*Spermophilus beldingi*), and California ground squirrels (*Spermophilus beecheyi*).

The monetary damage to range grasslands attributed to pocket gophers and ground squirrels is difficult to estimate. Rangeland rodents can reduce vegetation by 20%–30%, which results in less plant material available for livestock grazing (1,2). In addition, the combination of grazing by pocket gophers, ground squirrels, and livestock can lead to severe soil erosion. Damage to earthen irrigation ditches and dams has

been observed in areas where pocket gopher and ground squirrel populations have become excessive (1,2).

Methods for controlling ground squirrel and pocket gopher populations include exclusion, shooting, trapping, flooding, and use of acute toxicants including anticoagulants and fumigants (3). Fortified steam-rolled oat baits at 0.005% and 0.010% chlorophacinone or diphacinone are used in California grasslands to control rodent populations. Currently, these baits are registered exclusively for below-ground application. In anticipation of registering the baits for above-ground application by a broadcast method, chlorophacinone and diphacinone contamination of rangeland vegetation is a possibility. Since these same grasslands are commonly used for grazing by other herbivores, including livestock destined for commerce, it is necessary to assess trace levels of rodenticides present in range grass. To assay the residue levels of chlorophacinone and diphacinone in the range grass, an analytical method was developed and validated at concentrations of 0.1 to 10  $\mu\text{g}$  anticoagulant per gram of range grass. The validation range was determined through discussions with experienced field personnel that have used broadcast application methods for the California Department of Food and Agriculture (J. Clark and R. Eng, California Department of Food and Agriculture, personal communication, 1995).

A method for the analysis of chlorophacinone and diphacinone residues on vegetation was not located in the literature. Several methods have been developed for the analysis of indanediones in baits, formulations, and tissues. A gas chromatographic method with derivatization (5) is sensitive and selective but suffers from low recoveries and is time consuming. Spectrophotometric methods have been used (6,7) for baits and formulations but are not selective when multiresidue samples are assayed. Thin-layer chromatographic (8–10) methods are not suited for determining low levels of residues in complex matrices such as plant and animal tissues. Reversed-phase high-performance liquid chromatographic (HPLC) methods (11–15) provide sufficient sensitivity but often produce poor chromatographic resolution for the indanediones. Ion-pair reversed-phase HPLC (16–20) has demonstrated adequate sensitivity and selectivity but column lifetime is often short due to adsorption of the ion-pairing reagent onto the stationary phase of the column packing material. Reversed-phase

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ion-pair HPLC was chosen as the most appropriate method of analysis for our purposes because good chromatographic resolution can be maintained and the column can be preserved with regular column washing. The sample preparation procedure involved solid-phase extraction (SPE) with an aminopropyl sorbent that, to our knowledge, was previously unreported for the analysis of chlorophacinone and diphacinone residues. Our method had a limit of detection of 15 ppb or less.

## Experimental

### Reagents

Acetone, chloroform, ethyl acetate, methanol, and tetrahydrofuran (THF) were liquid chromatography grade (Fischer Scientific, Denver, CO). Deionized water was purified using a Milli-Q water purification system (Millipore, Bedford, MA). Concentrated phosphoric acid (Fischer Scientific) was used to make the 4N phosphoric acid in water.

Chlorophacinone (98.9%) was obtained from LiphaTech (Milwaukee, WI) and diphacinone (99.3%) was obtained from HACCO (Madison, WI). Concentrated stock standards of chlorophacinone and diphacinone were prepared by first drying the technical grade compounds for 4 h at 110°C and then dissolving 10.000 mg in 10.0 mL of ethyl acetate. Working standards that ranged in concentration from 0.030 to 10.0 µg/mL were prepared by dilution of stock solutions with mobile phase. All standard solutions were stored at 5°C.

Tetrabutylammonium dihydrogen phosphate (97%) was purchased from Aldrich (Milwaukee, WI) and was used to prepare a 5mM solution in methanol. A commercially prepared tetrabutylammonium dihydrogen phosphate ion-pairing reagent with a 0.05M potassium dihydrogen phosphate buffer was purchased from Alltech (Deerfield, IL) and was used to prepare a 5mM solution in water.

### Fortification of controls

Range grass was collected from a region of Stanislaus County, California with no known history of chlorophacinone and diphacinone use and designated the blank control material. The range grass from this part of California is a complex mixture of numerous plant species, the most abundant of which are soft chess (*Bromus mollis*), foxtail fescue (*Festuca megalura*), and broadleaf filaree (*Erodium botrys*) (J. Clark and R. Eng, California Department of Food and Agriculture, personal communication, 1995). Approximately 4 pounds of California range grass (control) was collected in early October. The California range grass was ground with a variable speed batch sample processor (Model RSI 6V, Robot Coupe U.S.A., Jackson, MS) and stored in a sealed stainless steel container. The method was validated at levels of 0.10, 1.0, and 10 µg/g chlorophacinone and diphacinone. Each 1.00–1.10-g portion of ground range grass was fortified with 10.0 µL of a 10.0, 100.0, or 1000 µg/mL standard solution in ethyl acetate to produce the appropriate fortification level.

### Extraction procedure

Ground range grass was accurately weighed in 1.00-g portions into 50-mL screwcap glass tubes. Then 10.0-mL of chloroform was pipetted into the sample tubes. The mixture was

vortex mixed for 10 s and then shaken horizontally with a mechanical shaker (Eberbach, Ann Arbor, MI) at high speed for 15 min. The sample tubes were sonicated in a beaker for three consecutive 15-min periods. The tubes were shaken by hand for a few seconds between each sonication period. The sample tubes were centrifuged at approximately 2500 rpm for 5 min.

### SPE cleanup and concentration

The extracts were filtered through a 0.45-µm Teflon syringe filter, and approximately 5.5–6.5 mL of the filtrate was collected in a glass tube. The 500-mg IST NH<sub>2</sub> (aminopropyl) SPE columns with a 10-mL reservoir (Jones Chromatography, Lakewood, CO) were conditioned with 4 mL of chloroform. The packing material was not allowed to dry. A 5.0-mL aliquot of filtered sample extract was added to each SPE column with a flow rate of approximately 3 mL/min. Each SPE column was rinsed with 10 mL of chloroform followed by 4 mL of ethyl acetate and 4 mL of methanol. The wash eluate was discarded. The SPE packing material was dried for approximately 5 min under vacuum or centrifugation. A 15-mL glass tube was placed under each SPE column in the manifold to collect the eluate containing the analytes. The analytes were eluted from each SPE column by the addition of 5 mL of the methanolic ion-pairing reagent in 1.0-mL increments.

The eluate of each sample was evaporated to dryness at 70°C under a gentle stream of nitrogen. The sample residues were reconstituted with 1.0 mL of mobile phase and sonicated for 5 min. The reconstituted samples were filtered through a 0.45-µm Teflon filter before injection into the HPLC.

### High-performance liquid chromatography

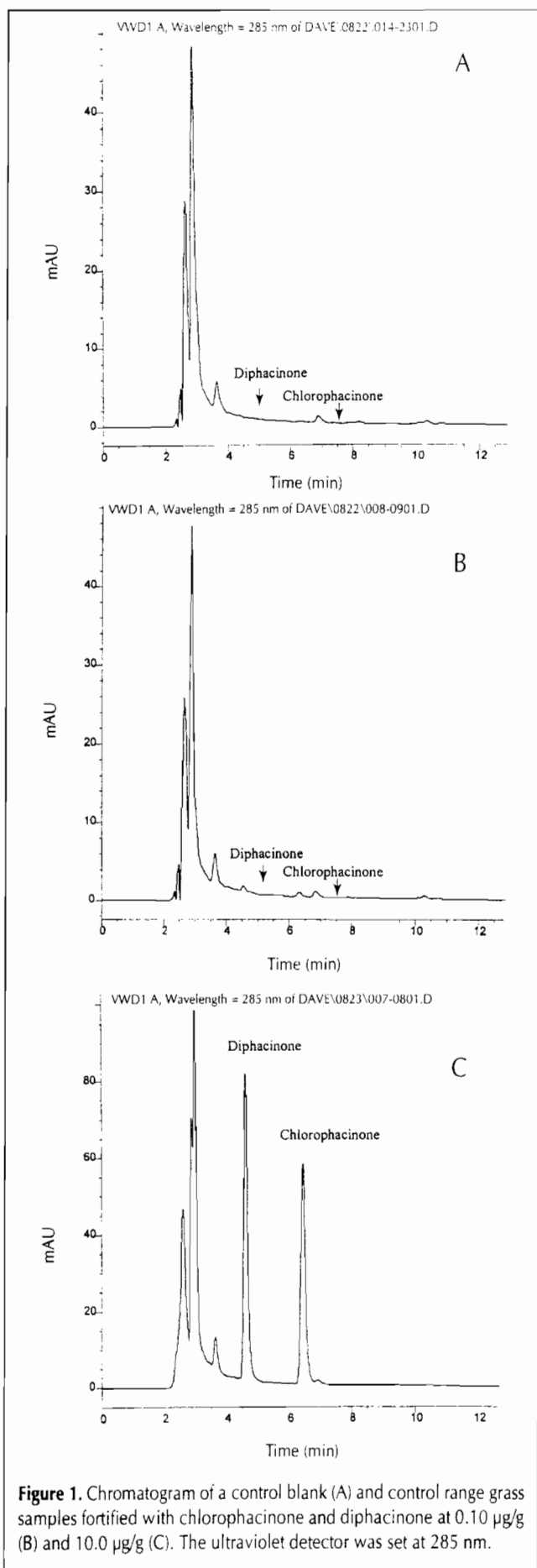
The HPLC system consisted of a Hewlett-Packard 1090 liquid chromatograph (Palo Alto, CA) operated at 35°C. A Hewlett-Packard 1050 variable wavelength detector at 285 nm was used to detect chlorophacinone and diphacinone. Aliquots of 25 µL were injected automatically by the pneumatically controlled injector valve. The analytes were separated on a 25-cm × 0.46-cm stainless steel analytical column packed with 5 µm Keystone ODS/H (Bellefonte, PA). The flow rate was 1.0 mL/min. To prolong column lifetime, a 1.5-cm × 0.46-cm i.d. Keystone ODS/H guard column was used. The mobile phase was prepared by mixing the aqueous and methanolic solutions of 5mM tetrabutylammonium dihydrogen phosphate in the ratio of 20:80 (v/v) and adjusting the pH to 7.5 with 4N phosphoric acid. The mobile phase was degassed by sparging with helium. At the end of each set of analyses, the column was washed with a mixture of 1:1 (v/v) methanol–water for 40 min.

For the chromatographic parameters chosen, the retention times of diphacinone and chlorophacinone were approximately 4.6 and 6.4 min, as shown in Figure 1.

## Results and Discussion

### Response linearity

Two sets of six calibration standard solutions were prepared; they ranged in concentration from 0.030 to 10 µg/mL. Each



**Figure 1.** Chromatogram of a control blank (A) and control range grass samples fortified with chlorophacinone and diphacinone at 0.10 µg/g (B) and 10.0 µg/g (C). The ultraviolet detector was set at 285 nm.

standard solution was injected twice, and linear regression was performed on the data set. The regression statistics are shown in Table I. A linear relationship exists between the peak response and the analyte concentration. The response is directly proportional to concentration over the range of interest. While the data indicate that single point calibrations were valid over this range of standard solution concentrations, a standard curve generated with four calibration standard solutions were used for assays at or near the limit of detection.

### Extraction

The extraction of diphacinone and chlorophacinone residues was accomplished by the extraction of ground samples with chloroform. Preliminary experiments were used to compare the extraction efficiency of residues from diphacinone- and chlorophacinone-fortified range with grass chloroform, acetone, chloroform-acetone (1:1, v/v), ethyl acetate, methanol, and THF. There were slightly higher recoveries when chloroform and chloroform-acetone were used. Extracts obtained with chloroform were cleaner than those obtained with all of the other solvents; therefore, chloroform was selected as the extraction solvent for this method.

### SPE cleanup

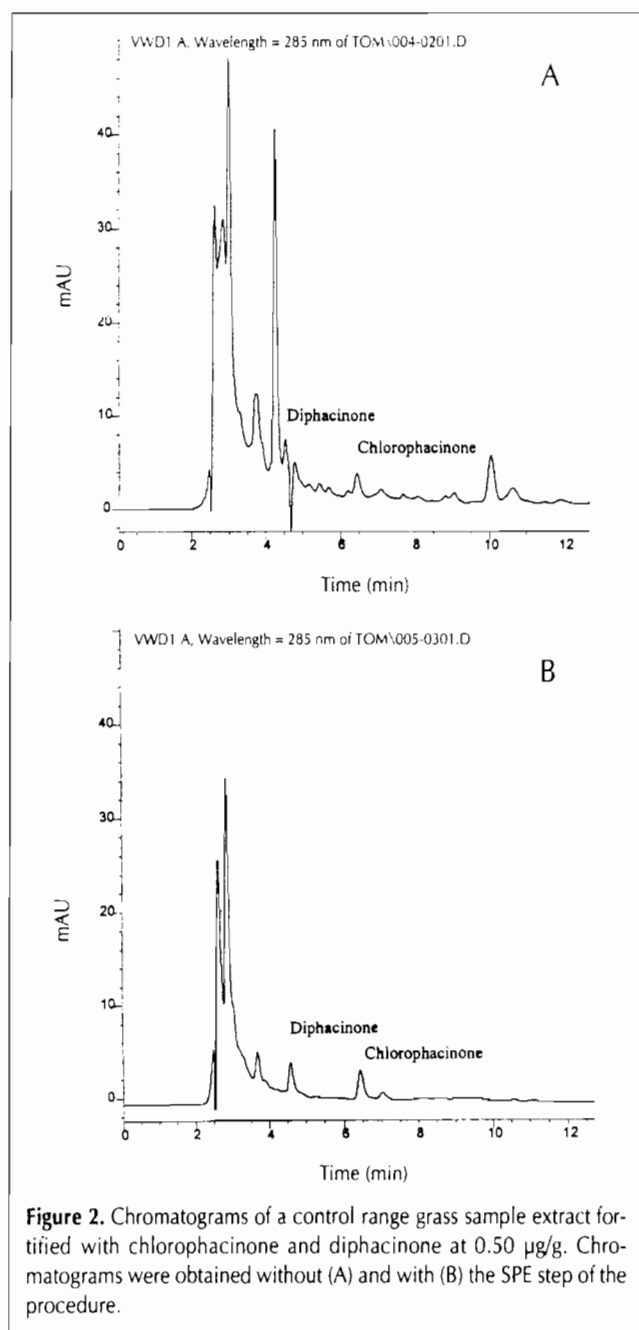
Based on the polar nature of the analytes and the solubility of the analytes in nonaqueous solvents, an SPE cleanup procedure was attempted by the adsorption of the analytes on NH<sub>2</sub>, 2,3-dihydroxypropyl (Diol), strong anion exchanger (SAX), florisil, and silica SPE columns. All the SPE columns were loaded, washed, and eluted by using the procedure described in the Experimental. The analytes were only partially retained on the SAX, silica, and florisil SPE sorbents during the washing steps. The only SPE sorbents to retain the analytes during the loading and washing procedure were the NH<sub>2</sub> and Diol sorbents. During the elution step, the analytes were partially retained on the Diol sorbent when eluted with the methanolic ion-pairing reagent. The analytes were completely eluted from the NH<sub>2</sub> sorbent. The NH<sub>2</sub> sorbent was adopted as the SPE column of choice for the remainder of the work.

Cleanup of the extract was completed in the following manner. The SPE column was washed with increasingly polar solvents (chloroform, ethyl acetate, and methanol). After elution of the analytes with the ion-pairing reagent in methanol, some of the matrix components were observed to remain on the

**Table I. Linear Regression Parameters**

Compound	R <sup>2</sup>	Slope	y-Intercept
<i>Peak area versus concentration</i>			
Diphacinone	.9993	143.980	0.309*
Chlorophacinone	.9998	127.895	0.989†
<i>Log of peak area versus log of concentration</i>			
Diphacinone	.9983	0.982	
Chlorophacinone	.9980	0.955	

\* Represents a concentration of 2 ppb.  
† Represents a concentration of 4 ppb.



**Figure 2.** Chromatograms of a control range grass sample extract fortified with chlorophacinone and diphacinone at 0.50 µg/g. Chromatograms were obtained without (A) and with (B) the SPE step of the procedure.

SPE column. Figure 2A is a sample chromatogram that was obtained with no SPE cleanup procedure. When compared with Figure 2B, which is a chromatogram of a sample after SPE cleanup, the benefits of the cleanup step can be observed. The SPE cleanup resulted in an overall decrease in the matrix components in addition to elimination of chromatographic interferences. The cleanup step also reduced the number of guard columns used. The method without the cleanup step would cause increased backpressure due to clogging of the guard column after approximately 35–40 injections of the sample. With the cleanup step, the guard column lifetime was extended to 65–70 injections of the sample.

#### Recoveries

The mean recoveries of chlorophacinone at the 0.10-, 1.0-, and 10.0-µg/g levels were 93.8% ± 3.0, 92.4% ± 3.9, and 95.4%

± 2.8. The mean recoveries of diphacinone at the 0.10-, 1.0-, and 10.0-µg/g levels were 101% ± 8.8, 94.5% ± 2.0%, and 96.9% ± 3.1%, respectively. Ten replicates were analyzed for each level by two analysts over an 8-day period with no significant interday differences observed. Two different lots of the NH<sub>2</sub> SPE columns were used to complete the method validation with no difference in recoveries observed between the two lots. Representative control samples (all components except diphacinone and chlorophacinone) were treated according to the procedures in this method. As can be seen in Figure 1A, no chromatographic responses were observed at or near the retention time of diphacinone in all control samples. Chromatograms of a fortified control grass sample (0.10 µg/g chlorophacinone and 10.0 µg/g diphacinone) are shown in Figures 1B and 1C for comparison.

#### Method limit of detection

The limit of detection was defined as the concentration of chlorophacinone or diphacinone required in the sample to generate a signal equal to 3 times the baseline noise (peak to peak) observed in the chromatogram of the control extract. The limit of detection was estimated from peak height of a control range grass extract and an extract from a control range grass sample fortified at 0.10 µg/g. Under the conditions specified in the method, the limit of detection was 0.015 µg/g for chlorophacinone and 0.013 µg/g for diphacinone.

#### Conclusion

The use of SPE with reversed-phase ion-pair HPLC was demonstrated to be an efficient method for the determination of chlorophacinone and diphacinone in range grass. Aminopropyl SPE columns have not previously been used for the assay of chlorophacinone and diphacinone in any matrix. To our knowledge, this is the first reported for the determination of the analytes in vegetation, as most previous methods involved the analysis of tissues, baits, formulations, and technical materials.

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Mention of commercial products is for identification only and does not constitute endorsement by the U.S. Government.

#### References

1. J.D. Chase, W.E. Howard, and J.T. Roseberry. In *Wild Mammals of North America*; J.A. Chapman and G.A. Feldhammer, Eds. The Johns Hopkins University Press, Baltimore, MD, 1982, pp 251–52.
2. P.Q. Tomich. In *Wild Mammals of North America*; J.A. Chapman

- and G.A. Feldhammer, Eds. The Johns Hopkins University Press, Baltimore, MD, 1982, pp 202–203.
3. T.P. Salmon and R.H. Schmidt. An introductory overview to California ground squirrel control. *Proc. Vert. Pest. Conf.* **11**: 32–37 (1984).
4. R.W. Bullard, R.D. Thompson, and G. Holguin. Diphenadione residues in tissues of cattle. *J. Agric. Food Chem.* **24**: 261–63 (1976).
5. Y. Kawano and W. Chang. Spectrophotometric determination of Rozol in paraffinized formulations. *J. Assoc. Off. Anal. Chem.* **63**: 996–98 (1980).
6. R.L. Caswell. Report of rodenticides. *J. Assoc. Off. Anal. Chem.* **42**: 104–106 (1959).
7. V. Mallet, D. Surette, and G.L. Brun. Detection of naturally fluorescent pesticides on silica gel layers. *J. Chromatogr.* **79**: 217–222 (1973).
8. P. Owen, A. Pendlebury, and A.C. Moffat. Choice of thin-layer chromatographic systems for the routine screening for neutral drugs during toxicological analyses. *J. Chromatogr.* **161**: 187–93 (1978).
9. K. Opong-Mensah and W.R. Porter. Separation of some rodenticides and related compounds by thin-layer chromatography. *J. Chromatogr.* **455**: 439–43 (1988).
10. J.B. Addison. Improved method for high pressure liquid chromatographic determination of chlorophacinone in mouse tissue. *J. Assoc. Off. Anal. Chem.* **65**: 1299–1301 (1982).
11. J.E. Houglum, R.D. Larson, and R.M. Neal. High-performance liquid chromatographic separation of indanedione rodenticides. *J. Chromatogr.* **481**: 458–60 (1989).
12. J.D. Reynolds. Extraction and identification of ten anticoagulant rodenticides from baits and stomach contents by high pressure liquid chromatography. *Proc. Am. Assoc. Vet. Lab. Diagn.* **23**: 187–94 (1980).
13. B.R. Bennett and G.S. Grimes. Reverse phase liquid chromatographic determination of chlorophacinone and diphacinone in bait formulations. *J. Assoc. Off. Anal. Chem.* **65**: 927–29 (1982).
14. P. Mura, A. Piriou, Y. Papet, D. Lochon, and D. Reiss. Rapid high-performance liquid chromatographic assay of chlorophacinone in human serum. *J. Anal. Toxicol.* **16**: 179–81 (1992).
15. Gy. Vigh, Z. Varga-Puchony, E. Papp-Hites, J. Hlavay, and S. Balogh. Determination of chlorophacinone in formulations by reversed-phase ion-pair chromatography. *J. Chromatogr.* **214**: 335–41 (1981).
16. K. Hunter. Reversed-phase ion-pair liquid chromatographic determination of chlorophacinone residues in animal tissues. *J. Chromatogr.* **299**: 405–14 (1984).
17. K. Hunter. High-performance liquid chromatographic strategies for the determination and conformation of anticoagulant rodenticide residues in animal tissues. *J. Chromatogr.* **321**: 255–72 (1985).
18. K. Hunter and E.A. Sharp. Modification to procedures for the determination of chlorophacinone and for multi-residue analysis of rodenticides in animal tissues. *J. Chromatogr.* **437**: 301–305 (1988).
19. T. Chalermchaikit, L.J. Felice, and M.J. Murphy. Simultaneous determination of eight anticoagulant rodenticides in blood serum and liver. *J. Anal. Toxicol.* **17**: 56–61 (1993).

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